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# *Colletotrichum fructicola*, first record of bitter rot of apple in China

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Abstract — *Colletotrichum fructicola*, isolated from diseased apple fruit with bitter rot symptoms collected in orchards of Henan Province, is a new record for China. It also represents a new pathogen species for apple bitter rot. Phylogeny inferred from combined data sets of the complete rDNA ITS1-5.8S-ITS2 region, partial actin (ACT), β-tubulin-2 (TUB2) and glyceraldehyde-3-phosphate dehydrogenase (GPDH) confirmed that the fungus should be included within *C. fructicola,* and this grouping was also congruent with morphology and culture characteristics.

Key words — anamorph, molecular, taxonomy, pathogenicity

## Introduction

A worldwide fungal disease, bitter rot of apple, can cause serious economic loss. Since 1990, the major pathogens causing bitter rot were reported as *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. and its teleomorph, *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk; *C. acutatum* J.H. Simmonds plays a relatively minor role (González et al. 2006).

*Colletotrichum* is one of the most economically important fungal genera (Hyde et al. 2009a). *Colletotrichum* species are cosmopolitan; multiple species can occur on a single host, and a single species is often found on multiple hosts (Cai et al. 2009). Sutton (1992) noted that morphology alone is not sufficient for a precise identification because the lack of reliable morphological features makes species boundaries ambiguous and confusing, especially in the *C. acutatum, C. gloeosporioides,* and *C. dematium* complexes. Reliable species identification is important for biosecurity, plant breeding, and integrated disease management.

Since 2009, MycoBank has recorded 28 new *Colletotrichum* species, including six previously considered to belong to the *C. gloeosporioides* complex. One species, *C. fructicola* isolated from coffee berries in Thailand, was separated from *C. gloeosporioides* complex by Prihastuti et al. (2009) based on multi-gene phylogeny and morphological characters.

In China, *C. gloeosporioides* was assumed to be the sole pathogen causing apple bitter rot (Wang & Li 1987) until Zhang et al. (2008) reported that *C. acutatum* could also cause the disease. In order to identify the *Colletotrichum* species causing bitter rot of apple in China, we collected and isolated the pathogen from diseased apple fruit in orchards in Henan Province in 2011 and 2012.

## Materials & methods

### Isolates & morphology

Apple orchards in Henan Province, China were surveyed for bitter rot symptoms in the autumn of 2011 and 2012. A small piece of decayed tissue was cut from arbitrarily selected lesions on fruits and disinfected with 70% ethanol before placed on potato dextrose agar (PDA). Four strains were isolated and incubated at 28°C in darkness for 7 days on PDA. A mycelial disc was then removed from the actively growing edge of a mono-conidial colony and transferred to new PDA. Three replicate cultures of each isolate were investigated. After 7 days at 25°C in darkness, size and shape of 50 conidia were measured (Than et al. 2008), and the colony diameter, conidial mass colors, and colony zonations were also recorded. Appressoria were produced using a slide culture technique, in which a 1 $cm^2$ segment of PDA containing the isolate was placed in sterile water in a sterile Petri dish, covered with a sterile cover slip, and incubated under high humidity at 25°C in darkness. After 2 days, the shape and size of 50 appressoria on the cover slip were recorded. The F11PGZH03 living culture was deposited in China General Microbiological Culture Collection Center (CGMCC), Beijing, China.

### DNA extraction & PCR sequencing

The protocol of Barnes et al. (2001) was used to extract genomic DNA from mycelium growing on PDA. The primers ITS1-F and ITS4 were used to amplify the ITS region of rDNA. The ACT gene was amplified with primer pair ACT512F and ACT783R; TUB2 with Bt2a and Bt2b; and GPDH with GDF1 and GDR1 (Prihastuti et al. 2009). The amplification conditions of PCR reactions were: initial denaturation at 95°C for 3 min followed by 34 cycles of denaturation at 95°C for 60 s, annealing at 52°C for 30 s, extension at 72°C for 1 min and a final cycle of 10 min at 72°C. The PCR products were sequenced by Sangon Biotech Co., Shanghai China, and the results were manually checked for errors.

### Phylogenetic analysis

Sequence data from ITS, ACT, TUB2, and GPDH genes of our isolate were deposited in GenBank and compared with other *Colletotrichum* sequences from GenBank

Table 1. *Colletotrichum* sequences used in the phylogenetic analysis

| Species / Strain* | GenBank accession numbers* | | | |
|---|---|---|---|---|
| | ITS | ACT | TUB2 | GPDH |
| *C. asianum* BML I3 [1] | FJ972605 | FJ903188 | FJ907434 | FJ972571 |
| **BPD I4** [1] | **FJ972612** | **FJ907424** | **FJ907439** | **FJ972576** |
| *C. falcatum* **FAL** [1] | **FJ972606** | **FJ907431** | **GQ289454** | **FJ972585** |
| *C. fructicola* BPD I12 [1] | FJ972611 | FJ907425 | FJ907440 | FJ972577 |
| **BPD I16** [1] | **FJ972603** | **FJ907426** | **FJ907441** | **FJ972578** |
| F11PGZH03 [2] | KC012513 | KC012505 | KC012517 | KC012509 |
| *C. gloeosporioides* **CBS 953.97** [1] | **FJ972609** | **FJ907430** | **FJ907445** | **FJ972582** |
| *C. hymenocallidis* **CSSN2** [3] | **GQ485600** | **GQ856775** | **GQ849438** | **GQ856757** |
| CSSN3 [3] | GQ485601 | GQ856776 | GQ849439 | GQ856759 |
| *C. kahawae* **IMI 319418** [1] | **FJ972608** | **FJ907432** | **FJ907446** | **FJ972583** |
| IMI 363578 [1] | FJ972607 | FJ907433 | FJ907447 | FJ972584 |
| *C. siamense* **BPD I2** [1] | **FJ972613** | **FJ907423** | **FJ907438** | **FJ972575** |
| BML I15 [1] | FJ972614 | FJ907422 | FJ907437 | FJ972574 |
| *C. simmondsii* **BRIP 28519** [4] | **FJ972601** | **FJ907428** | **FJ907443** | **FJ972580** |

*Type strains and sequences in bold font

[1] Prihastuti et al. (2009); [2] this paper; [3] Yang et al. (2009); [4] Hyde et al. (2009b).

(Table 1), including those of several type specimens (Hyde et al. 2009b, Prihastuti et al. 2009, Yang et al. 2009). Preliminary alignments of the four gene sequences were conducted using Clustal X (Thompson et al. 1997) with manual adjustment, and using BioEdit for visual improvement wherever necessary. Phylogenetic analysis of the combined ITS/ACT/TUB2/GPDH sequences was performed using PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2003). All characters were unordered and of equal weight and gaps were treated as missing data. Clade stability of the tree resulting from maximum parsimony analysis (MP) and neighbor-joining analysis (NJ) was evaluated by 1000 bootstrap replications on the same aligned dataset (Hillis & Bull 1993). Measures calculated for parsimony included tree length (TL), consistency index (CI), retention index (RI), and rescaled consistency index (RC). The outgroup was *Colletotrichum falcatum*.

**Pathogenicity testing**

The spores of isolate F11PGZH03 were harvested from PDA cultures, and the concentration of spore suspension in sterile water was adjusted to $10^6$ conidia/ml using a haemocytometer. Mature and non-wounded Fuji apple fruits were surface-sterilized with 70% ethanol prior to inoculation. Apples were inoculated by using wound/drop and non-wound/drop inoculation methods (Prihastuti et al. 2009). The isolate inoculation was repeated three times. Control fruits were treated similarly to the inoculated fruit, but with sterile water instead of spore suspension. After inoculation, the fruits were placed in a closed plastic bag to maintain high humidity and held at 25°C in the darkness. The length and width of typical symptoms on the skin of fruit were recorded after 5 and 10 days.

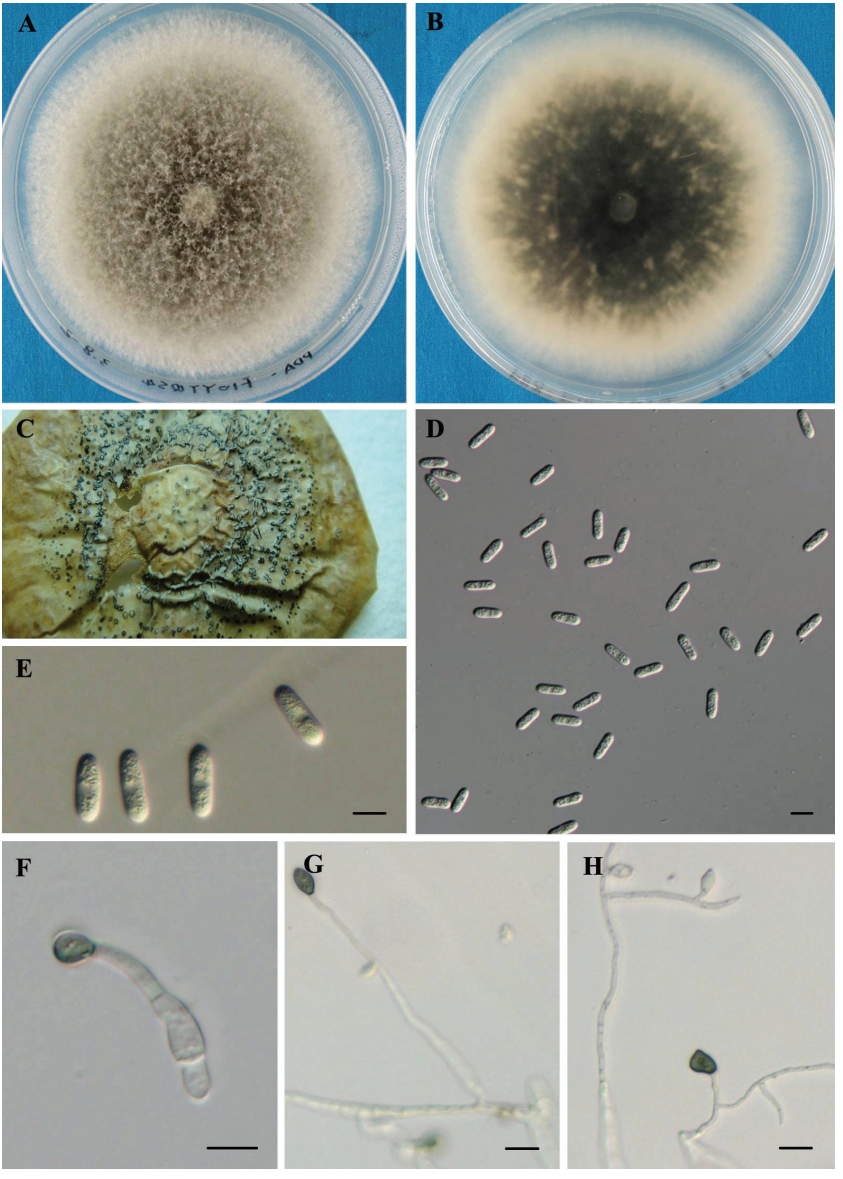


Fig. 1. *Colletotrichum fructicola* (F11PGZH03). A–B, colony on PDA after 7 days, (A = upper; B = reverse). C, bitter rot symptom on apple skin. D–E, conidia. F–H, appressoria. Bars: D–H = 10 μm.

## Results

### Taxonomy

Based on morphological, cultural, and multigene phylogenetic data, the isolates were identified as:

***Colletotrichum fructicola*** Prihastuti, L. Cai & K.D. Hyde, Fungal Diversity 39: 96. 2009. Fig. 1

Sexual morph not observed. Asexual morph on PDA. Hyphae hyaline, smooth-walled, septate, branched. Conidiophores formed directly on hyphae. Setae not observed. Conidia hyaline, smooth-walled, aseptate, straight, cylindrical with both ends round or one end slightly acute, (12.3–)1–3.5–15.5(–17.1) × (4.3–)4.7–5.5(–6.3) μm, mean ± SD = 14.59 ± 1.16 × 5.11 ± 0.38, L/W ratio = 2.9. Appressoria single or in loose groups, pale to dark brown, ovoid and slightly irregular to irregular in shape and often becoming complex with age, (6.4–)7–9(–10.5) × (4.7–)5.2–6.2(–6.9) μm, mean ± SD = 8.08 ± 1.03 × 5.78 ± 0.54, L/W ratio = 1.4.

Cultural characteristics — Colonies grown from single conidia on Difco PDA 78–80 mm diam after 7 d. Aerial mycelium white to pale grey, dense, cottony, surface of agar pale grey to dark grey towards centre, more of less white at the edge; acervuli not observed. In reverse grayish green towards centre with white halo. In 7 days at 28°C, growth rate 8.5–9.25 mm/day (mean ± SD = 9.06 ± 0.375, n = 9).

Specimens Examined: **China**, **Henan Province**: Zhengzhou City, on fruit surface of apple (*Malus domestica* Baumg.), 28 Sept. 2011, Dandan Fu F11PGZH03 (living culture, CGMCC 3.15207; GenBank KC012513, KC012505, KC012517, KC012509); Shangqiu City, 6 Sept. 2012, F12PGSQ5; F12PGSQ6; Xiayi County, 7 Sept. 2012, F12PGXY1.

### Phylogenetic analysis

Of 1863 characters aligned with Clustal X and optimized manually to allow maximum alignment and maximum sequence similarity by BioEdit, 246 were parsimony informative. Ambiguously aligned regions were excluded from all analyses. The Kishino-Hasegawa (KH) test showed that three trees generated from parsimony analysis were retained. One of the most parsimonious trees is shown in Fig. 2 (TL = 867, CI = 0.9331, HI = 0.0669, RI = 0.8578, RC = 0.8005). MP and NJ bootstrap values are provided above branches. The phylogram in Fig. 2 illustrates that our isolate F11PGZH03 grouped with two *C. fructicola* isolates in a single clade with 100%/100% bootstrap support, indicating that they may represent the same species.

In addition to isolating F11PGZH03, we isolated three strains of *C. fructicola* in Henan Province in 2012. The grouping of these three strains was well supported by phylogenetic analysis of ITS sequences and multilocus sequence

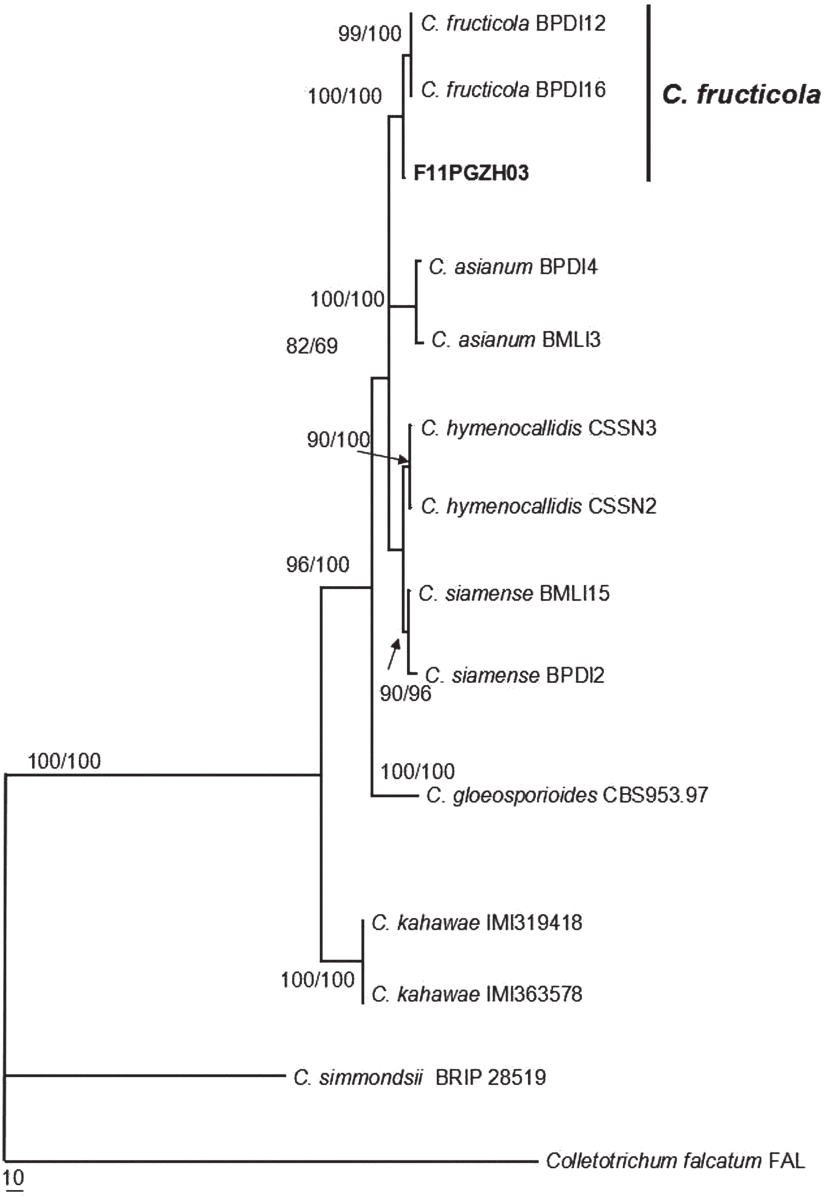


Fig. 2. Phylogram generated from maximum parsimony and neighbor-joining analyses based on combined ITS, ACT, TUB2, and GPDH sequence data. Clade stabilities shown above branches were calculated from maximum parsimony (>50%) and distance (>50%). The scale bar shows 10 changes. The tree is rooted with *Colletotrichum falcatum*.

data (results not shown). The colonies, conidia, and appressoria of our isolates were morphologically distinguishable from those of *C. gloeosporioides* complex.

### Pathogenicity tests

All four isolates produced typical symptoms of bitter rot in Fuji apples by wound inoculation, except for isolate F11PGZH03, which did not produce rot symptoms after non-wound inoculation. Symptoms produced by each isolate were similar, and there were no apparent differences in lesion size and characters among isolates.

## Discussion

Based on phylogenetic analysis of combined ITS/ACT/TUB2/GPDH datasets and morphological characters of the anamorph, we identified our isolates as *Colletotrichum fructicola,* which represent a new species record for the genus in China.

*Colletotrichum fructicola* was previously described only from coffee berries and leaf spots of peanut (Prihastuti et al. 2009). In this study we isolated the fungus from apple, which is a newly reported host for *C. fructicola.* Based on the pathogenicity test, *C. fructicola* was also determined to be a bitter rot pathogen of apple fruit.

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